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Inoculating chlamydospores of *Trichoderma asperellum* SM-12F1 changes arsenic availability and enzyme activity in soils and improves Water Spinach growth

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Abstract: Arsenic (As)-contaminated agricultural soils threaten crop yields and pose a human health risk. Augmentation of exogenous microorganisms exhibiting plant-growth promoting and As speciation changing shows potential to improve crop growth and change soil As availability. *Trichoderma asperellum* SM-12F1 exhibiting both traits was developed into chlamydospores to improve its persistence in contaminated soils. After inoculation, As availability and enzyme activity in two types of soils and the growth as well as As uptake of water spinach (*Ipomoea aquatic* Forsk.) were investigated. The results indicated that inoculation significantly improved water spinach growth in both soils. Inoculating chlamydospores at 5% significantly increased As concentration (139%), bioconcentration factor (150%), and translocation factor (150%) in water spinach grown in Chenzhou (CZ) soils, while no significant change for these in Shimen (SM) soils. Inoculating chlamydospores at 5% caused a significant increase (16%) of available As content in CZ soils, while a significant decrease (13%) in SM soils. Inoculation significantly caused As methylation in both soils, while significant As reduction merely observed in CZ soils. The differential changes in available As contents in both soils were attributed to the soil pH, As fractionations and speciation characteristics. Furthermore, Inoculating chlamydospores at 5% significantly improved the activities of β-glucosidase (155%), chitinase (211%), and phosphatase (108%) in SM soils, while significant decreases in β-glucosidase (81%), phosphatase (54%), aminopeptidase (60%), and catalase (67%) in CZ soils. Bioaugmentation and As availability change were responsible for this result. These observations will be helpful for the application of fungal chlamydospores in the future bioremediation.

Keywords: Fungi; Arsenic; Water spinach; Soil enzyme; Speciation; XANES

40 **1. Introduction**

41 Arsenic (As) accumulates in soil via various natural routes, such as rock weathering and volcanic
42 activity, as well as from anthropogenic activities that include mining/smelting, crop irrigation and
43 pesticide/preservative use (Abernathy et al., 2003). There is mounting concern regarding human
44 exposure to As through soil-plant transfer, in addition to more established exposure risks from
45 drinking/cooking water supplies. Arsenic's negative effects on human health from chronic
46 exposure are widespread, but it's most notable as a potent carcinogen (Fayiga and Ma, 2006). In
47 agronomic settings, As has historically been used extensively as a herbicide and defoliant
48 (Williams et al., 2005). Impacting on various metabolic processes, As causes physiological and
49 morphological disorders leading to reduced plant growth (Tripathi et al., 2013; Zhao et al., 2010).
50 It disrupts also, the regulation of essential nutrients within plant tissues, reducing the overall
51 quality of crops as a food source (Williams et al., 2007). *In solum*, As can depress soil enzyme
52 activity, a commonly used metric for biological diversity, ecosystem functioning and overall soil
53 fertility (Marx et al., 2001; DeForest, 2009), which can also depress plant growth.

54

55 The discovery and effective utilization of microorganisms capable of improved As
56 tolerance/detoxification combined with plant-growth promotion (PGP) characteristics to mitigate
57 crop plant exposure to As and enhance productivity are highly prized technologies (Das et al.,
58 2014). Recent studies showed that arbuscular mycorrhizal fungi inoculation can improve As
59 tolerance in tomato (Hua et al., 2009), plantain (Or owska et al., 2012), Chinese brake fern (Leung
60 et al., 2010), and medic (Zhang et al., 2015). Arsenic-resistant bacteria have also been
61 successfully screened/selected for PGP (Cavalca et al., 2010; Shagol et al., 2014; Ghosh et al.,

2015). It is well known that microbial excretion of hormones and/or enhanced nutrient supplementation play a role in PGP (Khan et al., 2009; Lampis et al., 2015). Comparatively, however, more research attention has been paid to the application of PGP by arbuscular mycorrhizal or bacteria. Far less is known about the potential of filamentous fungi as agents of PGP for the remediation of As-contaminated soils (Babu et al., 2014a). Filamentous fungi have a distinct advantage over bacteria because of their high tolerance to As and other metals/metalloids and their abilities to grow under extreme conditions of pH, temperature and nutrient availability (Anand et al., 2006). More importantly, some filamentous fungus can be developed into chlamydospores, with asexual reproduction and thick cell walls that confer additional protection enabling them to further tolerate contaminated soils (Lewis and Papavizas, 1983). Additionally, fungal chlamydospores can be easily developed into solid powders that are convenient for storage, and improve the delivery of the inoculum into the soil.

Some PGP microorganisms can trigger soil As speciation change (ASC), subsequently changing As phytoavailability. Das et al. (2016) observed that PGP *Bacillus flexus* ASO-6 can oxidize arsenite (As(III)) and reduce As-uptake by rice and promote plant growth in As-stressed soil. Xu et al. (2016) found that root or leaflet endophytes exhibiting plant-growth promoting traits can resist As via arsenate (As(V)) reduction, promote the growth of As-hyperaccumulator *Pteris vittata* and increase phytoremediation efficiency. These microorganisms that exhibit both PGP and ASC traits show great potential as biotechnology tools for promoting crop yields, whilst simultaneously changing plant As uptake. Unfortunately, the study of these PGP-ASC organisms in As-contaminated soils is scarce.

84

85 Soil enzyme activities are known to be affected by As, heavy metals and microbial biomass
86 (Bhattacharyya et al., 2008). Low enzymatic activities have been observed in soils heavily
87 contaminated with toxic trace metals (Hagmann et al., 2015). Koo et al. (2012) observed that
88 water soluble As especially, exerts a strong inhibitory effect on the soil enzyme activities. The
89 microbial biomass is the major source of enzymes in soil and is highly susceptible to disruption by
90 As and heavy metal contamination (Boshoff et al., 2014). The augmentation of exogenous
91 microorganisms capable of As speciation change could enhance the microbial biomass (Tripathi
92 et al., 2015) as well as labile As content in soil (Wang et al., 2015), which subsequently influences
93 soil enzyme activities. Thus, enzyme activity analysis can be helpful in assessing the biochemical
94 quality of As-contaminated soils after microbial inoculation.

95

96 Recently, *Trichoderma asperellum* SM-12F1 has been reported for its abilities of As resistance and
97 speciation transformation (Zeng et al., 2010; Su et al., 2011, 2012). It has been well reported that
98 *Trichoderma* greatly contributes to PGP, biological control, and modification of plant metabolism
99 (Harman et al., 2004). Babu et al. (2014b) observed that *Trichoderma* spp. can enhance host
100 plant growth through production of Indole acetic acid (IAA), 1-aminocyclopropane-1-carboxylic
101 acid (ACC) deaminase, siderophores, or acid phosphatase under biotic and abiotic stresses.
102 Furthermore, *T. asperellum* SM-12F1 was successfully developed into chlamydospores to improve
103 its persistence in contaminated soils (Wang et al., 2015). In this study, pot experiments were set
104 up by inoculating with chlamydospores of *T. asperellum* SM-12F1 into two types of As-enriched
105 soils with different contamination sources (mining vs. industrial). Water spinach (*Ipomoea*

106 *aquatic* Forsk.), which is a popular vegetable crop in As-contaminated areas of southern China,
107 was selected as the test plant species. The objectives were to determine: (I) the growth and As
108 uptake of water spinach after inoculation; (II) the reproduction of *T. asperellum* SM-12F1 and
109 changes in soil enzymes activities; (III) variances in As availability, fractionation as well as
110 speciation in soils after inoculation. To our best knowledge, this is the first report to exploit the
111 application of fungal chlamydospores capable of plant-growth promotion and As speciation
112 transformation in As-contaminated soils.
113
114 **2. Materials and methods**
115 *2.1 Preparation of the chlamydospores of T. asperellum SM-12F1 and soil samples*
116 The As-resistant fungal strain, *T. asperellum* SM-12F1, was isolated from a slag heap near the
117 realgar mine in Shimen county of Hunan province, China. The speciation transformation of As by *T.*
118 *asperellum* SM-12F1 had been well investigated (Zeng et al., 2010; Su et al., 2011, 2012).
119 Following our previous procedure (Wang et al., 2015), *T. asperellum* SM-12F1 was successfully
120 cultivated into chlamydospores via a chlamydospores production medium and then developed
121 into solid powder after grinding the air-dried culture residue. The pH of the chlamydospores
122 powder (5.78) was determined potentiometrically at a 1: 2.5 ratio of powder to ultrapure water
123 prepared by using Milli-Q water purification system (Millipore Corporation, USA). No As in
124 chlamydospores powder was detected out using hydride generation atomic fluorescence
125 spectrometer (HG-AFS 9120, Titan instrument, Beijing, China).
126
127 Two types of experimental soils were collected from the As-contaminated field soils in Chenzhou

City (CZ soils) and Shimen County (SM soils) of Hunan province, China, respectively. The former (Calcari-Leptic Cambisol) developed from the limestone is adjacent to a timber yard where arsenide is often used in wood preservation, while the latter (Alumi-Plinthic Acrisol) developed from quaternary red clay is located at the downstream of a realgar mine. Both sampling sites were contaminated after As enriching soils via flood moving. After air-dried, grounded, and passed through the 2-mm sieve, the experimental soils were mixed thoroughly. The physic-chemical properties of soils are listed in Table S1 (in the Supplementary Data).

2.2 Pot experiment

The pot experiment was conducted in a glasshouse of Chinese Academy of Agricultural Sciences. For each experimental soil (SM and CZ), chlamydospores powder (1.2×10^7 cfu g^{-1} obtained via the dilution plate method) of *T. asperellum* SM-12F1 with three levels (m/m) of 0% (CK), 1.0%, or 5.0% were inoculated. In order to provide enough carbon and nitrogen sources for fungal survival, glucose (2.0%) and asparagines (0.5%) were spiked into each soil (Sneh et al., 1984). Furthermore, each soil received the same amount of chemical fertilizers (0.72 g kg^{-1} of $CO(NH_2)_2$, 0.32 g kg^{-1} of KH_2PO_4, and 0.54 g kg^{-1} of K_2SO_4) to meet the demands of plant growth modified from the report of Hseu et al. (2013). After complete mixing, each soil was transferred into a pot. Four replications were run for each inoculation level. Six seeds of water spinach purchased from Beijing Ju Hong Seed Technology co., LTD were surface sterilized with H_2O_2 (5%) and then sown in each pot. Subsequently, the seedlings were thinned to three per pot after germination. Soil moisture content was maintained at 60% of field capacity during the germination and then adjusted to field capacity after thinning. The temperature in the glasshouse was maintained at

30 ℃ during the daytime and 25 ℃ at night. All pots were arranged in a completely randomized design.

2.3 Biomass analysis and survival number of T. asperellum SM-12F1 determination

Water spinach was harvested 2 months after sowing. The height of water spinach was measured by using a stainless steel ruler with accuracy of 0.01cm. The shoot and root of water spinach were separated and subsequently weighted after being dried at 65℃ for 48 h. The plant samples were ground using a stainless steel grinder. For each treatment, fresh soils samples were harvested and divided into three sub-samples. The first (20.0 g) of which was used to determine the survival number of *T. asperellum* SM-12F1 using the dilution plate method and the selective medium (Supplementary information Fig. S1) modified from the descriptions of Elad and Chel (1983) and Papavizas (1982). The second (10.0 g) was immediately stored at -20℃ for As speciation analysis. Finally, the last portion was air-dried and then ground to pass 2 mm sieve for subsequent analysis.

2.4 Analysis of As contents in soil or plant samples

For plant samples, each shoot or root sample of 1.000 g was digested by mixed HNO_3 of 20 ml, H_2SO_4 of 1.25 ml, and $HClO_4$ of 1 ml until the digestion solution was clear (PRC National Standard, GB/T 5009.11-1996). After filtration and volume fixation at 50 ml, the total As content was measured using HG-AFS. During HG-AFS analysis, the mixture of 1% ascorbic acid, 1% thiocarbamide, and 3% hydrochloric acid was used to preliminarily reduce sample As into As(III). The mixture of 0.5% potassium hydroxide and 1% potassium borohydride was used to further

reduce As(III) into AsH_3 and subsequently As content was determined. 3% hydrochloric acid was used as sample carrier. In order to characterize As uptake and assimilation patterns in water spinach, bioconcentration factor (BCF) and translocation factor (TF) were measured. The BCF of As in water spinach was calculated by the ratio of As concentrations in shoot and soil (Zhuang et al., 2007). The TF of As was calculated from the ratio of As concentrations in shoot and root (Yoon et al., 2006).

For soil samples, soil available As was extracted with 0.5 M $NaHCO_3$ (Woolson et al., 1971). A soil sample of 5.00 g was suspended in 0.5 M $NaHCO_3$ of 50 ml and then shaken for 2 h at room temperature. Subsequently, the soil suspension was filtered before As determination by HG-AFS. An improved sequential extraction proposed by Wenzel et al., (2001) was adopted to determine As fractionation. The five sequential extraction steps were assumed to correspond respectively to non-specifically sorbed As (F1), specifically sorbed As (F2), As associated with amorphous and poorly-crystalline hydrous oxides of Fe and Al (F3), As associated with well crystallized hydroxides of Fe and Al (F4), and residual As (F5). Additionally, soil pH was also measured potentiometrically at a 1:2.5 ratio of soil to H_2O after 1 minute of shaking.

2.5 In situ analysis of As speciation in soils using in-situ X-ray absorption near edge structure (XANES)

Arsenic speciation analysis of fresh soil samples using in-situ XANES was conducted at beam line 15U1 of Shanghai synchrotron radiation facility. Sub-samples of fresh soil, after defrosting at room temperature, were fixed to Mylar membranes (thickness of 6 μm) that was fixed onto a

sample table for analysis. For each sample, three sites were randomly selected for XANES analysis. Each site was scanned for 6 s using a spot size of 3.18 × 2.56 μm^2 from 11,850 to 11,900 eV with a 0.5 eV step size. To correct for the effect of the synchrotron radiation beam flux variation on signal intensity, the fluorescence intensity was normalized to the incident X-ray intensity, which was monitored using an ionization chamber located in front of the K-B mirror modulating the size of the beam (Zheng et al., 2011). The corrected fluorescence intensity was used to estimate the relative elemental content. During XANES analysis, As standard compounds were prepared with high-purity (>94.5%) chemicals including As(III), $NaAsO_2$ (Riedel-de Haen AG, Seelze-Hannover, Germany); As(V), $Na_2HAsO_4{\cdot}7H_2O$ (Dr. Ehrenstorfer, Germany); MMA, CH_4AsNaO_3 (Dr. Ehrenstorfer, Germany); and DMA, $C_2H_6AsNaO_2$ (Dr. Ehrenstorfer, Germany).

2.6 Analysis of soil enzymes using microplate fluorimetry

Activities of hydrolase enzymes such as β-glucosidase, aminopeptidase, and acid phosphatases are the keys controlling the availabilities of C, N, and P in soil, respectively (Rejmánková and Sirová, 2007). Chitinase is essential in the mineralization of N from chitin as a main component of fungal cell wall (Olander and Vitousek, 2000). Catalase is one of the important antioxidant enzymes contributing to the detoxification of reactive oxygen species generated due to heavy metal stress (Patel et al., 2016). In this study, the activities of the β-glucosidase, chitinase, and acid phosphatase were measured fluorometrically using MUB (methylumbelliferone)-linked model substrates (DeForest, 2009). While for aminopeptidase and catalase, L-Leucine-7-amino-4-methylcoumarin and L-DOPA (3, 4-dihydroxy phenylalanine) were used as substrates. Model substrates and operation procedure in detail were listed in Table S2. Enzyme

activities were expressed in units of nmol h^{-1} g^{-1} and calculated in the method from DeForest (2009).

2.7 Quality assurance and control

For As concentration analysis using HG-AFS, As standards were prepared using As stock solutions (GBW08611, Chinese Metrology Institute of Science and Technology, Beijing, China). The correlation coefficients of the obtained linear equation reached 0.9993. A certified reference material (CRM) water-sample (GBWZ50004-88, Institute for Environmental Reference Materials, Ministry of Environmental Protection, Beijing, China), elemental spikes, and blanks were incorporated as part of stringent quality control protocols, which were analysed at the beginning, after every 10 samples and at the end of each run.. Furthermore, every sample was measured in triplicate. To verify the digestion procedures, Spinach CRM (GBW10015, Institute of geophysical and geochemical exploration, Chinese Academy of Geological Sciences) was incorporated into every sample batch. The accuracy of the As measurement of the spinach CRM ranged from 99-108%. The overall accuracy of the soil As fractionation procedure, as determined by comparing the sum of As determined in all five fractions with a single total As determination, was found to be within the range of 86-113%.

2.8 Statistical analysis

All experimental data was processed with Microsoft Excel 2003 and expressed with mean ± standard error (SE). Tukey test was applied to the one-way analysis of variance ($P < 0.05$) with the use of SPSS 16.0 software (SPSS Inc., Chicago, IL). For in-situ XANES analysis, the relative weight of

each As speciation was obtained via linear combination fitting (LCF) of the XANES spectra with model compounds using IFEFFIT software. The R-factor representing the goodness-of-fit parameter was calculated. Good fits occur for $R < 0.05$. In this study, R-factor was below than 0.01 at each fitting.

3. Results

3.1 Biomass and As contents of water spinach grown in soils after inoculation

Inoculating with chlamydospores of *T. asperellum* SM-12F1 significantly improved the biomass of water spinach (Table 1). For CZ soils, when inoculation level reached 5%, the height, shoot dry weight, and root dry weight of water spinach significantly ($P < 0.05$) increased by 35%, 216%, and 87%, respectively, compared with the control. For SM soils, compared with control, the height, shoot dry weight, and root dry weight of water spinach significantly ($P < 0.05$) increased by 64%, 141%, and 148%, respectively, when inoculation level reached 5%. When both soils at equivalent inoculation levels were compared, water spinach biomass in SM soils was significantly ($P < 0.05$) higher that in CZ soils.

Inoculating with chlamydospores of *T. asperellum* SM-12F1 at different soil loadings affected both As uptake and assimilation by water spinach, a trend observed in both soils (Table 2). For CZ soils, when inoculation level reached 5%, As concentration (7.75 mg kg^{-1}) and contents (19.92 μg pot^{-1}) in the shoot of water spinach significantly ($P < 0.05$) increased by 139.2% and 637.8% compared with their control. While no significant change was observed for As concentration and content in root. For SM soils, when inoculation level reached 5%, the As concentration (98.83 mg kg^{-1}) in

root significantly (P < 0.05) decreased by 15% compared with the control (115.99 mg kg^{-1}). However, the As content (178.60 μg pot^{-1}) in root significantly (P < 0.05) increased by 100.6% compared with the control (89.05 μg pot^{-1}), due to the higher dry weight of root. No significant change for As concentration and content in shoot was observed after inoculation. Furthermore, for BCF and IF of As, no significant difference was found in SM soils after inoculation. However, the BCF and IF in CZ soils both significantly (P < 0.05) increased by 150% compared with the control, when chlamydospores were inoculated at 5%. Inoculating with chlamydospores at 5% into CZ soils considerably promote the As translocation in water spinach.

3.2 Fungal augmentation and soil available As content after inoculation

The successful augmentation of *T. asperellum* SM-12F1 in both soils was observed after inoculation with chlamydospores (Fig S1 in the Supplementary Data). For both soils without inoculation, the survival number of *T. asperellum* SM-12F1 was about 47.5-60.0 cfu g^{-1} fresh soils. When chlamydospore was inoculated at 1%, the survival numbers of *T. asperellum* SM-12F1 reached 1.4 $\times 10^4$ and 7.4$\times 10^6$ cfu in fresh CZ and SM soils, respectively. When inoculation level of chlamydospores increased to 5%, the survival numbers of *T. asperellum* SM-12F1 were 1.6 $\times 10^5$ and 5.0$\times 10^8$ cfu in fresh CZ and SM soils, respectively. The augmentation of *T. asperellum* SM-12F1 in SM soils was more effective than that in CZ soils.

Inoculating with chlamydospores of *T. asperellum* SM-12F1 differentially changed the contents of available As in both soils (Fig 1). When inoculation level reached 1% and 5%, the contents of available As were 6.5 and 6.6 mg kg^{-1} in CZ soils, respectively, which significantly (P < 0.05)

increased by 14.1% and 16.0% compared with that in control (5.7 mg kg^{-1}). For SM soils, however, no significant change was found for available As when inoculation level was 1%. While the content of available As (4.5 mg kg^{-1}) significantly ($P < 0.05$) decreased by 13% compared with that in control (5.2 mg kg^{-1}).

3.3 Soil pH and As fractionations after inoculation

Inoculation with chlamydospores was responsible for a decreasing in soil pH (Fig S2). For both soils, no significant change in soil pH was found after inoculation with chlamydospores at 1%. However, when inoculation level reached 5%, the pH values in CZ and SM soils decreased to 6.6 and 5.0 from 7.3 and 5.6 in their corresponding controls, respectively. Comparatively, the pH values in SM soils were lower than in the CZ soils. This could be explained by the lower original pH in SM soils and better survival of *T. asperellum* SM-12F1, which are know to release organic acids.

Inoculating with chlamydospores of *T. asperellum* SM-12F1 changed the As fractionation patterns of the soils (Table S3). For both experimental soils, F3 and F2 fractions dominated, which accounted for approximate 35% and 26% of the total, respectively. Furthermore, with increasing inoculation level, no significant change was found in the As concentration of the F4 and F5 fractions in CZ soils. When inoculation level of chlamydospores reached 5%, As content in F1 (1.07 mg kg^{-1}) significantly ($P < 0.05$) increased by 98% while no significant change was found in As contents in F2 or F3, compared with their corresponding controls. For SM soils, however, no significant variance was observed for As content in F3, F4, or F5 among different inoculation

304 levels. When inoculation level of chlamydospores reached 5%, As content in F1 (1.47 mg kg^{-1})
305 significantly ($P < 0.05$) increased by 72.9% while that in F2 (11.4 mg kg^{-1}) significantly ($P < 0.05$)
306 decreased by 61.4%, compared with their corresponding controls. Changes in the contents of
307 non-specifically (F1) and specifically (F2) sorbed As in both soils after inoculation might be
308 responsible for the variances in available As.
309
310 *3.4 As speciation in soils after inoculation measured using in situ XANES*
311 Inoculating with chlamydospores of *T. asperellum* SM-12F1 significantly changed the As
312 speciation and promoted As methylation in both soils. The XANES spectra corresponding to each
313 soil sample and the spectra for standards of As(III), As(V), MMA, and DMA are presented in Fig S3.
314 Evaluation of the XANES spectra beyond the absorption edge shows differences in the region of
315 11,865-11,875 eV among three fungal strains. The relative weight of each As speciation was
316 obtained via LCF of the XANES spectra with model compounds (Fig 2). For both soils without
317 inoculation, the dominant species was As(V) with trace amounts of As(III) present, which
318 accounted for 84-86% and 14-15% of the total, respectively. For CZ soils, after inoculating with
319 chlamydospores of 1%, As(III), As(V), and MMA accounted for 46%, 46%, and 8% of the total,
320 respectively. When inoculation level reached 5%, As(III), As(V), and MMA accounted for 44%,
321 37%, and 20.0% of the total, respectively. Comparing the As speciation among different
322 treatments, the relative weight of As(III) significantly ($P < 0.05$) increased after inoculation.
323 Correspondingly, the relative weight of As(V) significantly ($P < 0.05$) decreased and MMA
324 emerged. For SM soils, however, after inoculating with chlamydospores at 1%, As(III), As(V),
325 MMA, and DMA accounted for 26%, 54%, 17%, and 3% of the total, respectively. When

inoculation level reached 5%, As(III), As(V), and MMA accounted for 19%, 64%, and 17% of the total, respectively. Comparing the As speciation trends among different treatments, no significant change was observed for As(III) or As(V). While organic As species, of MMA and DMA emerged after inoculation, indicating that microbial inoculation was enhancing As methylation in both soils.

3.5 Soil enzymes after inoculation measured using microplate fluorimetry

Inoculating with chlamydospores of *T. asperellum* SM-12F1 significantly and differentially changed the enzyme activities in two types of soils (Fig 3). For CZ soils, chlamydospores inoculation lowered the activities of enzymes to different extents. When inoculation level reached 5%, the activities of β-glucosidase (43 mmol g^{-1} h^{-1}), phosphatase (463 mmol g^{-1} h^{-1}), aminopeptidase (57 mmol g^{-1} h^{-1}), and catalase (298 mmol g^{-1} h^{-1}) significantly ($P < 0.05$) decreased by 81%, 54%, 60%, and 67% compared with these in controls, respectively. While no significant change was found for chitinase activities. For SM soils, when inoculation level of chlamydospores reached 5%, the activities of β-glucosidase (3163 mmol g^{-1} h^{-1}), chitinase (5700 mmol g^{-1} h^{-1}), and phosphatase (3100 mmol g^{-1} h^{-1}) significantly ($P < 0.05$) improved by 155%, 211%, and 108% compared with these in the controls, respectively. While no significant change was found for the activities of catalase and aminopeptidase activity significantly decreased after inoculation.

4. Discussion

4.1 Inoculating with chlamydospores of T. asperellum SM-12F1 promotes the growth of water

spinach

Trichoderma has been extensively exploited in agriculture for plant growth promotion, biological control, modification of plant metabolism (Hoyos-Carvajal et al., 2009), environmental bioremediation (Wang et al., 2015). *T. asperellum* exhibited some plant growth-promoting attributes of phosphate solubilization, ACC deaminase activity, auxin, and siderophore production (Qi and Zhao, 2013). This might be responsible for the growth improvement of water spinach (Table 1). Furthermore, the activities of phosphatase, chitinase, and β-glucosidase in SM soils were improved considerably when 5% of chlamydospores inoculated (Fig 3). This might contribute to the growth of water spinach. For CZ soils, however, significant decreases in enzyme activities except for chitinase were found after inoculation. Further analysis showed that the activities of β-glucosidase (R^2=0.6309, n=12, $P < 0.01$), phosphatase (R^2=0.5738, n=12, $P < 0.01$), aminopeptidase (R^2=0.3694, n=12, $P < 0.05$), and catalase (R^2=0.3336, n=12, $P < 0.05$) significantly and negatively correlated with the available As contents in CZ soils. The increased labile As in soils could inhibit the activities of soil enzymes (Bhattacharyya et al., 2008; Liang et al., 2014).

Water spinach growth also might be influenced by As phytotoxicity. In this case, significant increase in As contents of water spinach shoot in CZ soils was observed and while no significant change was found in SM soils after inoculation (Table 2). However, the water spinach biomass greatly increased in both soils. Arsenic speciation in biomass might be changed after inoculation, which sequentially lowered the phytotoxicity of As. This is supported by the observation that inoculation resulted in the emergence of organic As species in the soil, which is regarded as a

370 detoxification process (Fig 2). Tripathi et al. (2015) suggested that As methylation occurred in soils
371 after inoculating *Trichoderma* could alleviate As stress in chickpea. The result from Cattani et al.
372 (2015) showed that inoculating *Rhizophagus irregularis* changes As speciation and toxicity in
373 maize shoot but didn't alter total As concentrations. Based on the above mentioned, in our
374 opinion, the plant growth might be determined by the synergetic effects of the plant
375 growth-promoting traits of *T. asperellum* and the changes in soil available As contents, soil
376 enzyme activities, and As phytotoxicity.
377
378 *4.2 Inoculation with chlamydospores of T. asperellum SM-12F1 changes As availability in soils*
379 The changes of soil pH, As fractionations, and chemical valence can greatly influence As
380 availability in soils (Quazi et al., 2011). In this study, available As contents significantly increased
381 in CZ soils while they decreased in SM soils after inoculation (Fig 1). Linear regression analysis
382 indicated that there was a significantly positive relationship ($Y=0.545X+1.9067$, $R^2=0.41$, $P < 0.01$)
383 between the available As contents in SM soils (Y) and soil pH (X). The lower pH might favor the
384 decrease of As availability in soils via ligand exchange reactions or electrostatic interactions with
385 soil minerals (Dixit and Hering, 2003). For CZ soils, however, it was difficult to explain the changes
386 of As availability based on soil pH.
387
388 As fractionations in soils can greatly affect As availability. In order to better explain the
389 relationship between As fractionations (X) and available As contents (Y), a stepwise regression
390 equation was applied to the CZ soils: Available $As=0.068+2.187F1+0.076F3$ ($R^2=0.9506$, $P < 0.01$).
391 Comparatively, As in F1 was the dominant factor for As availability change, due to its higher

coefficient than F3 (0.076). This means that the increase of non-specially absorbed As was responsible for the augment of available As contents in CZ soils. For SM soils, a stepwise regression equation could also be derived: Available As=4.135+0.023F2 (R^2=0.7191, P < 0.01). This relationship indicates that the significant decrease in available As in SM soils was due to changes to the pool of As associated with amorphous and poorly-crystalline hydrous oxides of Fe and Al.

Arsenic speciation transformation can change As availability in soils. After inoculation, the relative weight of As(III) in CZ soils was significantly higher than that without inoculation (Fig 2). This might be helpful in explaining the increased availability As in CZ soils. Because As(III) is not absorbed as strongly to soil as As(V), and hence has a greater mobility (Chatain et al., 2005). For SM soils, however, no significant change was observed for in the proportion of As(III) or As(V) among treatments. Importantly, *T. asperellum* SM-12F1 inoculation caused As methylation in both soils (Fig 2). This was consistent with the results of Tripathi et al. (2015), who found that MMA and DMA contents increased in rhizosphere soils of chickpea after being inoculated with *Trichoderma*. Organic As is seemed with less toxicity and mobility than inorganic As (Akter et al., 2005). Methylation will lower the toxic As stress to water spinach growth and change As availability in soils. In this study, XANES method is used to determine As speciation in soil samples rather than the typical method. Because XANES with the advantages of requiring no sample preparation and chromatographic separation, has been certified to be a valuable and reliable tool to detect As speciation (Su et al. 2015; Zeng et al. 2015).

414 *4.3 Future application of T. asperellum SM-12F1 chlamydospores in remediation of*
415 *As-contaminated soils*
416 *T. asperellum* SM-12F1 inoculation showed differential effects on As uptake and transfer in water
417 spinach between two types of soil. For CZ soils, the water spinach biomass, As content in shoot,
418 BCF, and TF significantly improved after inoculation (Table 2). It might be feasible to improve the
419 bioremediation efficiency by inoculating *T. asperellum* SM-12F1 in CZ soils. The results from
420 Lampis et al. (2015) indicated that inoculation with growth-promoting rhizobacteria increased
421 biomass of hyperaccumulator *Pteris vittata* by up to 45% and increased As removal efficiency
422 from 13% without bacteria to 35%. For SM soils, however, water spinach biomass significantly
423 increased while no significant change in As contents of shoot, BCF, and TF after inoculation was
424 observed (Table 2). It is recommended to inoculate *T. asperellum* SM-12F1 into SM soils where
425 planted with crops with the lower ability to uptake As or bioenergy crops such as maize or
426 sugarcane. The results from Cattani et al. (2015) showed that inoculation with *Rhizophagus*
427 *irregularis* in combination with phosphorus application could augment the maize biomass but
428 make no effect on total As content in shoot. Babu et al. (2014a) suggested that inoculation with *T.*
429 *virens* PDR-28 is beneficial for heavy metal phytostailization and maize biomass production as a
430 potential source of bio-fuel in the quest for renewable energy.
431
432 **5. Conclusions**
433 *T. asperellum* SM-12F1 inoculation significantly promoted the growth of water spinach. However,
434 the effects on As uptake and transfer in water spinach and As availability varied between two
435 types of As-contaminated soils. Inoculation significantly increased the As content and BCF as well

as IF of As in water spinach and As availability in CZ soils, while no significant change for these items was found in SM soils. These observations will be helpful for the future application of *T. asperellum* SM-12F1 chlamydospores in the bioremediation of different As-contaminated soils.

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Table 1 Heights and biomass weights of water spinach grown in As-contaminated Chenzhou (CZ) and Shimen (SM) soils inoculated with different levels of chlamydospores of *T. asperellum* SM-12F1.

Treatments	*Water spinach*		
	Height cm	Shoot dry weight g pot^{-1}	Root dry weight g pot^{-1}
CZ soils			
Control	12.2±0.6 b	0.8±0.1 c	0.6±0.1 b
1%	14.6±0.9 b	1.5±0.2 b	0.7±0.1 ab
5%	16.4±0.7 a	2.6±0.2 a	1.1±0.1 a
SM soils			
Control	25.2±0.7 b	2.5±0.4 b	0.8±0.1 b
1%	41.4±2.0 a	4.6±0.7 a	1.1±0.2 b
5%	41.1±1.0 a	5.9±0.3 a	1.7±0.2 a

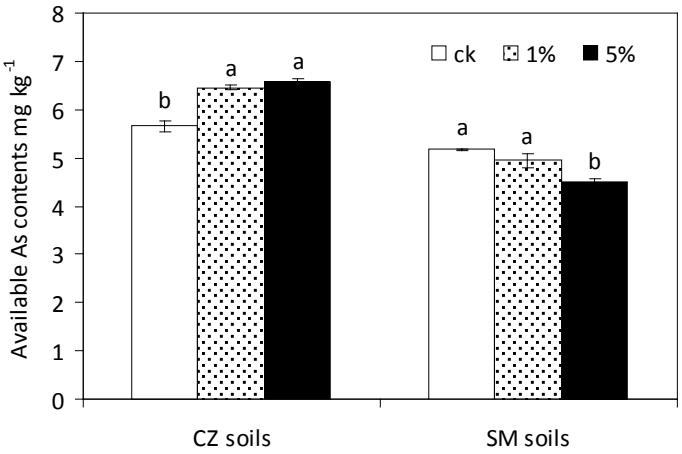
The different lowercase letter indicates significant difference ($P < 0.05$) in heights or biomass weights among different inoculation levels in an individual soil. Data is shown as average value ± standard error (SE).

Table 2 Arsenic content and bioconcentration factors (BCF) as well as translocation factors (TF) of As in water spinach grown in As-contaminated Chenzhou (CZ) and Shimen (SM) soils inoculated with different levels of chlamydospores of *T. asperellum* SM-12F1.

Treatments	As in shoot of *Water spinach*		As in root of *Water spinach*		BCF	TF
	mg kg^{-1}	μg pot^{-1}	mg kg^{-1}	μg pot^{-1}		
CZ soils						
Control	3.2±0.5 b	2.7±0.6 b	52.7±4.7 a	32.3±9.2 a	0.02±0.00 b	0.06±0.01 b
1%	4.4±1.0 b	7.0±2.0 b	55.9±6.5 a	38.7±7.2 a	0.03±0.01 b	0.08±0.02 b
5%	7.7±0.7 a	19.9±2.6 a	52.9±1.6 a	59.8±9.0 a	0.05±0.01 a	0.15±0.01 a
SM soils						
Control	2.4±0.4 a	6.2±1.7 a	116.0±18.0 a	89.1±18.4 b	0.02±0.00 a	0.02±0.00 a
1%	2.6±0.2 a	12.0±2.5 a	127.0±14.0 a	128.0±16.0 ab	0.02±0.00 a	0.02±0.00 a
5%	2.6±0.4 a	12.8±2.3 a	98.8±8.9 b	178.6±14.3 a	0.01±0.00 a	0.02±0.00 a

The different lowercase letter indicates significant difference ($P < 0.05$) in As concentrations (mg kg^{-1}), contents (μg pot^{-1}), BDF, or IF among different inoculation levels in an individual soil. Data is shown as average value ± standard error (SE).

Fig 1.



637 **Fig 2.**

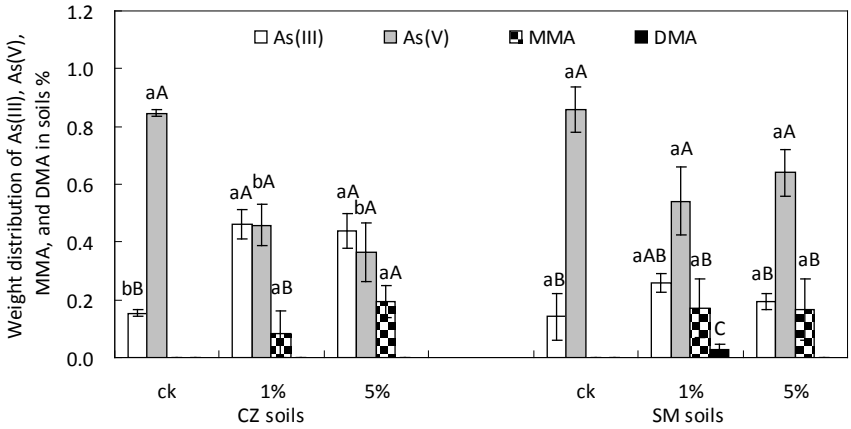


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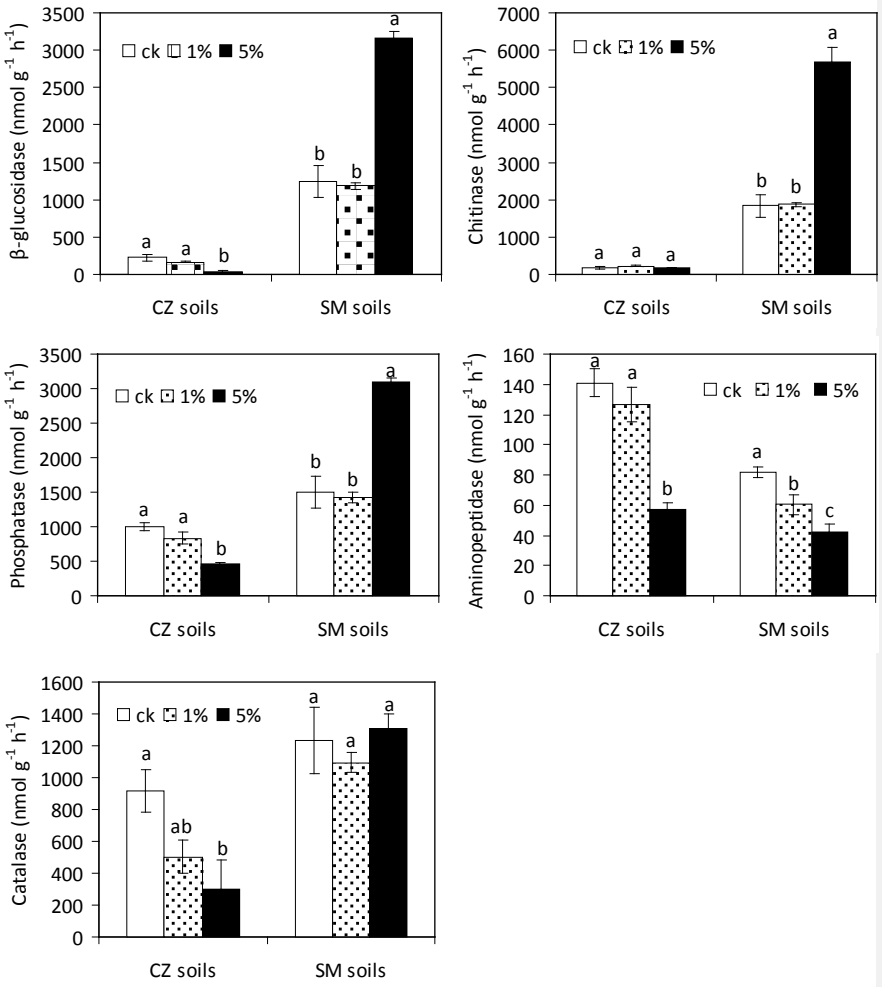
Fig 3.


β-glucosidase (nmol g-1 h-1)
Chitinase (nmol g-1 h-1)
Phosphatase (nmol g-1 h-1)
Aminopeptidase (nmol g-1 h-1)
Catalase (nmol g-1 h-1)
ck 1% 5%
CZ soils
SM soils